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Distinct roles for Mediator Cdk8 module subunits in *Drosophila* development

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Mediator (MED) is a conserved multisubunit complex bridging transcriptional activators and repressors to the general RNA polymerase II initiation machinery. In yeast, MED is organized in three core modules and a separable 'Cdk8 module' consisting of the cyclin-dependent kinase Cdk8, its partner CycC, Med12 and Med13. This regulatory module, specifically required for cellular adaptation to environmental cues, is thought to act through the Cdk8 kinase activity. Here we have investigated the functions of the four Cdk8 module subunits in the metazoan model *Drosophila*. Physical interactions detected among the four fly subunits provide support for a structurally conserved Cdk8 module. We analyzed the *in vivo* functions of this module using null mutants for *Cdk8*, *CycC*, *Med12* and *Med13*. Each gene is required for the viability of the organism but not of the cell. Cdk8–CycC and Med12–Med13 act as pairs, which share some functions but also have distinct roles in developmental gene regulation. These data reveal functional attributes of the Cdk8 module, apart from its regulated kinase activity, that may contribute to the diversification of genetic programs.

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Introduction

Cell fate specification during development is ensured by the progressive deployment of a great variety of DNA-bound transcription factors that control gene expression (Davidson *et al*, 2003). Much of the specificity of this process occurs at the pre-initiation step of transcription. There, an evolutionarily conserved complex of ~25 subunits termed Mediator (MED) plays a pivotal role in the fine-tuned recruitment of the general RNA polymerase II (PolII) initiation machinery to gene promoters (Kornberg, 2005). Indeed, it is now widely accepted that MED integrates and conveys regulatory signals by bridging specific activators and repressors to PolII and associated general transcription factors (GTFs) (Bjorklund and Gustafsson, 2005; Conaway *et al*, 2005; Kim and Lis, 2005; Malik and Roeder, 2005).

The structural and functional organization of MED has been well characterized in the budding yeast *Saccharomyces cerevisiae* (Bjorklund and Gustafsson, 2005). It is composed of three core modules (Kang *et al*, 2001; Guglielmi *et al*, 2004) that can interact with an additional, separable regulatory module consisting of a cyclin-dependent kinase (Cdk), Cdk8, its C-type cyclin (CycC) partner, Med12 and Med13 (Borggrefe *et al*, 2002). This interaction appears to be transient, as a recent genome-wide analysis of MED subunit localization on chromosomal DNA revealed that the Cdk8 module occupies the same sites as core MED but at generally lower levels (Andrau *et al*, 2006). The four yeast Cdk8 module subunits display similar loss-of-function phenotypes that reflect their shared specific requirements for cellular adaptation to environmental stresses, notably nutrient deprivation and heat shock (Carlson, 1997). A *CDK8* missense mutant that inactivates the kinase activity without affecting its incorporation into MED provokes the same defects as a deletion allele (Liao *et al*, 1995; Borggrefe *et al*, 2002), and affects the transcription of the same gene subset (Holstege *et al*, 1998). It has therefore been proposed that all functions of the four Cdk8 module components are mediated by the kinase activity of Cdk8 (Myer and Young, 1998). A comparative genome-wide analysis revealed that each of the four subunits regulates essentially the same genes (van de Peppel *et al*, 2005). Although most target genes are subjected to repression, some are upregulated by Cdk8 module activity. The regulatory function of Cdk8 kinase involves phosphorylations of the carboxy-terminal domain (CTD) of the large PolII subunit, other MED subunits, GTFs and gene-specific activators (Hengartner *et al*, 1998; Hirst *et al*, 1999; Chi *et al*, 2001; Vincent *et al*, 2001; Nelson *et al*, 2003; Hallberg *et al*, 2004; Liu *et al*, 2004; van de Peppel *et al*, 2005). Both reconstituted Cdk8–CycC pair and purified Cdk8 module can phosphorylate the CTD *in vitro* (Liao *et al*, 1995; Borggrefe *et al*, 2002). Thus, yeast Med12 and Med13 are apparently not essential for Cdk8 catalytic activity, raising questions about their precise roles within the Cdk8 module.

All four subunits of the *S. cerevisiae* Cdk8 module have structural counterparts in the distantly related fission yeast *Schizosaccharomyces pombe* and in metazoans (Borggrefe *et al*, 2002; Boube *et al*, 2002; Samuelsen *et al*, 2003), suggesting that the architecture of this regulatory MED module has been conserved during evolution. Direct support for this hypothesis is provided by the isolation of functionally distinct *S. pombe* and mammalian MED forms simultaneously harboring, or lacking, Cdk8, CycC, Med12 and Med13 (Conaway *et al*, 2005; Malik *et al*, 2005). Consistent with a conserved repressive role for the Cdk8 module, purified mammalian MED that contains Cdk8 harbors little

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or no PolII, whereas isolated core complexes are associated with near stoichiometric amounts of PolII subunits (Sato *et al*, 2004; Malik *et al*, 2005). Thus, metazoan Cdk8 module may inhibit stable PolII–MED interaction, as in yeasts (Hengartner *et al*, 1998; Elmlund *et al*, 2006). Finally, Med12 depletion in human cells results in reduced levels of Cdk8 protein, in the cell and within MED (Kim *et al*, 2006).

The physiological roles of Cdk8 module subunits in eukaryotes other than fungi have only recently begun to come to light. In the slime mold *Dictyostelium discoideum*, both *Cdk8* and *Med13* mutants are unable to form multicellular aggregates upon nutrient deprivation (Kon *et al*, 2000; Takeda *et al*, 2002). In the worm *Caenorhabditis elegans*, *Med12* and *Med13* mutants show similar defects of the female vulva and male tail (Wang *et al*, 2004; Yoda *et al*, 2005). Finally, *Drosophila melanogaster Med12* and *Med13* have indistinguishable loss-of-function phenotypes in eye and wing morphogenesis (Treisman, 2001; Janody *et al*, 2003). These genetic studies collectively suggest that metazoan Med12 and Med13 act together within a Cdk8 module. However, a comparative functional analysis of Cdk8, CycC, Med12 and Med13 has not yet been performed in any metazoan.

Drosophila represents an appropriate genetic model to examine, *in vivo*, the functional relationships of the four Cdk8 module components in a higher eukaryote. Here, we find that *D. melanogaster* Cdk8 and CycC can physically interact with Med12 and Med13, reinforcing the idea that these conserved subunits retain a Cdk8 module architecture from yeast to metazoans. To examine the developmental roles of the fly Cdk8 module subunits, we have generated null alleles of *Cdk8* and *CycC* and compared their effects with previously described loss-of-function alleles of *Med12* and *Med13.* All four genes are essential for the development of the organism but not for cell viability. Consistent with a paired action of Cdk8 and CycC *in vivo*, mosaic adults harboring clones of $Cdk8^{-}$ or $CycC^{-}$ cells exhibit indistinguishable defects in leg, eye and notum differentiation. However, although the mutant phenotypes for *Cdk8–CycC* closely resemble those for *Med12–Med13* in some situations, they diverge significantly in others. These effects on adult morphology are corroborated at the level of gene expression for several developmentally important genes, including *decapentaplegic*, *dachshund*, *bric-à-brac-2* and *senseless*, whose expression patterns are differentially affected, according both to the tissue and the mutated subunit. Our results reveal that Med12 and Med13 can have specific roles distinct from cyclin-regulated Cdk8 activity and thus underline the functional diversity of Cdk8 module subunits during development.

Results

Drosophila Cdk8 and CycC interact physically with Med12 and Med13

Biochemical studies from yeast, mammalian and *Drosophila* cells that identified distinct forms of MED containing or lacking the four Cdk8 module subunits Cdk8, CycC, Med12 and Med13, have suggested that the Cdk8 module architecture has been conserved during evolution (Bjorklund and Gustafsson, 2005; Conaway *et al*, 2005; Kim and Lis, 2005; Malik and Roeder, 2005). Consistent with this, *D. melanogaster* Cdk8 and CycC interact both *in vitro* and *in vivo* (Leclerc *et al*, 1996). Similarly, based on their identical mutant phenotypes and co-immunoprecipitation from extracts of embryos overexpressing both proteins, *Drosophila* Med12 and Med13 have been proposed to function as a unit *in vivo* (Treisman, 2001; Janody *et al*, 2003). To examine the possibility of direct binding between fly Med12 and Med13 with Cdk8 and CycC, we used the glutathione *S*-transferase (GST) pull-down assay. GST-Cdk8 and GST-CycC fusion proteins were expressed in bacteria and purified from total cell extracts by affinity on glutathione beads (Figure 1A). Under relatively stringent conditions, GST-CycC specifically interacted with *in vitro* translated ^{35}S-labeled Cdk8 (Figure 1B), as previously shown (Leclerc *et al*, 1996). In addition, GST–Cdk8 bound to radiolabeled Med12 as well as Med13 (Figure 1B, upper part), whereas GST–CycC did not (Figure 1B, upper part). However, under slightly less stringent conditions, GST–CycC also interacted with Med12 and Med13 (Figure 1B, lower part).

To provide an independent test for direct interactions between the four fly subunits, each was fused to the yeast Gal4 DNA-binding (G_{BD}) or activating (G_{AD}) domain, and then tested in the two-hybrid system for interactions with the other three subunits. As shown in Figure 1C, G_{BD}-fused CycC interacted with Cdk8, Med12 and Med13. However, interactions were not detected when CycC was fused to the G_{AD} as well as when G_{BD}- or G_{AD}-fused Cdk8 was tested with CycC, Med12 or Med13 (not shown). Such negative results are not necessarily informative, as similar situations are often encountered even where crystallographically demonstrated protein–protein contacts exist. As summarized in Figure 1D, our new results reinforce and extend previous data to support a structurally-conserved metazoan Cdk8 module, where each subunit is in contact with the three others.

Cdk8 and CycC are essential for development but not for cell viability

To examine the developmental functions of the *Drosophila* Cdk8 module, we sought to compare mutants for each subunit. Although null alleles of *D. melanogaster Med12* and *Med13* have been described (Treisman, 2001; Janody *et al*, 2003), no mutants for *Cdk8* or *CycC* were known. We therefore employed imprecise excision of nearby *P* transposons to generate *Cdk8* and *CycC* loss-of-function mutations. *Cdk8* alleles were generated by excising a homozygous viable *P*-element insertion situated 328 base pairs (bp) from the 3′ end of Cdk8 mRNA, in the 5′-untranslated region (UTR) of the neighboring *I-2* gene (Figure 2A, upper part). One recessive lethal allele chosen for subsequent analyses, $Cdk8^{K185}$, retains the *P* extremity in *I-2* and deletes 882 bp, including the C-terminal one-third of *Cdk8* protein-coding sequences. This allele causes lethality in late third-instar larvae (L3) and behaves as a null in complementation test with a larger deletion (see Supplementary data). Further, homozygotes for $Cdk8^{K185}$ are fully rescued to yield viable, morphologically normal fertile adults by a *Ub*-Cdk8 transgene that ubiquitously expresses normal Cdk8 protein (see Figure 2B, and not shown). Finally, wild-type Cdk8 protein was not detected in Western analyses of ventral nerve cords and associated imaginal discs prepared from mutant L3 tissues (Figure 2C).

To obtain loss-of-function *CycC* alleles, we similarly generated imprecise excisions of a viable insertion in its 5′-UTR region (Figure 2A, lower part). One recessive lethal allele,

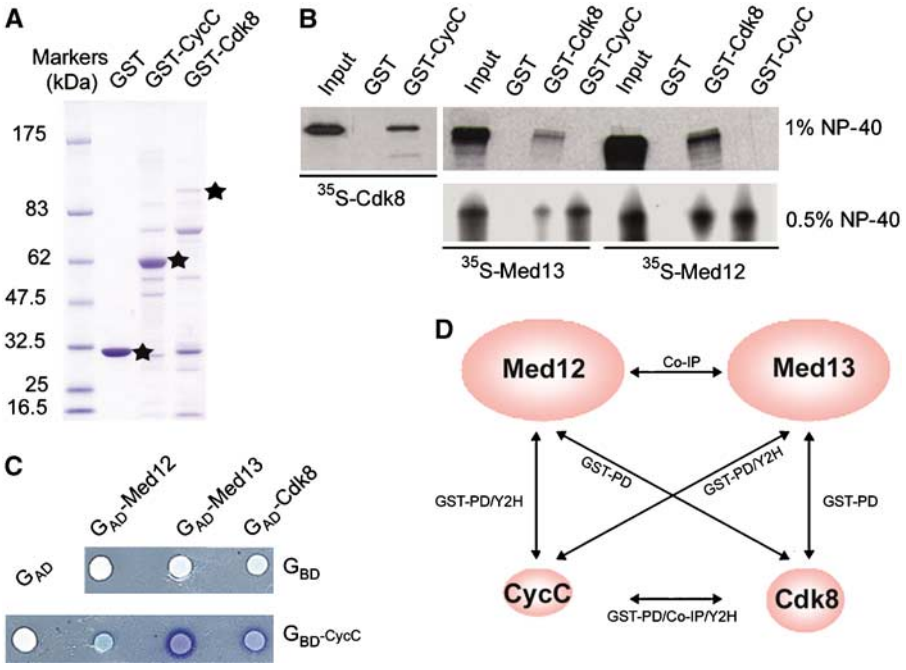


Figure 1 *Drosophila* Med12 and Med13 interact with Cdk8 and/or CycC *in vitro* and in a yeast two-hybrid assay. (**A**) Coomassie staining of purified recombinant GST-Cdk8 and GST-CycC fusions. Full-length forms are indicated by asterisks. (**B**) GST pull-down interactions between Cdk8, CycC, Med12 and Med13. ^{35}S-labeled Med12 or Med13 produced *in vitro* was incubated under two distinct conditions (1 or 0.5% NP-40) with GS-bound GST, GST-CycC or GST-Cdk8 (shown in panel A). Input (10%) and retained proteins were resolved by SDS–PAGE and detected by fluorography. (**C**) CycC interacts with Cdk8, Med12 or Med13 in a yeast two-hybrid assay. Interactions of CycC with Med12, Med13 or Cdk8 were revealed using an X-gal overlay assay as described in Werner *et al* (1993). Empty pAS2 vector (G_{BD}) or expressing a G_{BD}-CycC fusion was tested against empty pACT2 vector (G_{AD}) or expressing G_{AD}-Med12, G_{AD}-Med13 or G_{AD}-Cdk8 fusions as indicated. (**D**) Pairwise interactions between *Drosophila* Cdk8 module subunits, as detected from GST pull-down (GST-PD), yeast two-hybrid (Y2H) and/or co-immunoprecipitation (co-IP) experiments. Co-IP data are from Leclerc *et al* (1996) and Janody *et al* (2003).

CycC^{Y5}, deletes the entire *P* insert plus 1429 and 1304 bp of 5′- and 3′-flanking genomic DNA, respectively (see Figure 2A). Thus, *CycC*^{Y5} removes all *CycC* protein-coding sequences plus parts of its overlapping 5′- and 3′-gene neighbors, *CG3731* and *CG3641*. *CycC*^{Y5} is lethal at early pupal stage and, consistently with molecular data, behaves as a null allele based on complementation test with a larger chromosomal deficiency (see Supplementary data). *CycC*^{Y5} homozygotes could be rescued to a limited extent through a combination of *arm*-GAL4 and *UAS*-CycC transgenes (*arm>CycC*) that directs ubiquitous CycC expression (Figure 2B and Material and methods), although emerging adults showed some loss of sensory organs and malformed sex combs (not shown, and see below). This partial rescue was not enhanced on adding a *Ub*-CG3731 transgene and/or a wild-type copy of *CG3641* (*CycC*^{Y5}/*CycC*^{Y8} allele combination; see Figure 2B and Supplementary data), suggesting that the loss of *CG3641* or *CG3731* activity is not important. By contrast, adding a second *arm>CycC* copy led to extensive rescue (see Figure 2B), and such adults were morphologically normal (not shown). Finally, no CycC protein was detected in Western blot analyses from mutant L3 tissues (Figure 2C). We conclude that *Cdk8*^{K185} and *CycC*^{Y5} are null alleles whose effects are solely due to the loss of Cdk8 and CycC activity, and we refer to them in the remainder of the text as *Cdk8*$^{-}$ and *CycC*$^{-}$.

The developmental effects of these new *Cdk8* and *CycC* mutants were then compared with those of the characterized null alleles *Med12*^{T241} and *Med13*^{T606} (Treisman, 2001). All four mutants are recessive lethal, showing that each Cdk8 module component is required for the viability of the organism. However, *Med12* and *Med13* homozygous animals die as late embryos/early first-instar larvae, whereas *Cdk8*$^{-}$ and *CycC*$^{-}$ mutants survive until late L3/early pupae. Given that mRNAs encoding each Cdk8 module subunit are maternally contributed to the embryo (see FlyBase), this discrepancy might reflect their differential quantities and/or perdurance. Alternatively, Cdk8 module subunits might be required for divergent developmental processes. To avoid the complicating effects of maternal pools which partially compensate for zygotic mutations in early development, we used mitotic recombination to generate clones of homozygous mutant at later stages, where normal proteins are no longer detected (see Figure 2C). Clones of *Cdk8*$^{-}$ or *CycC*$^{-}$ cells were readily detected in all examined L3 imaginal discs (see below). This indicates that neither gene is required for cell viability, as is also the case for *Med12* and *Med13* (Boube *et al*, 2000; Treisman, 2001). Taken together, these results show that each Cdk8 module subunit is required for the development of the organism, but is dispensable for cell viability.

Shared and divergent roles for Cdk8 module subunits in distal leg development

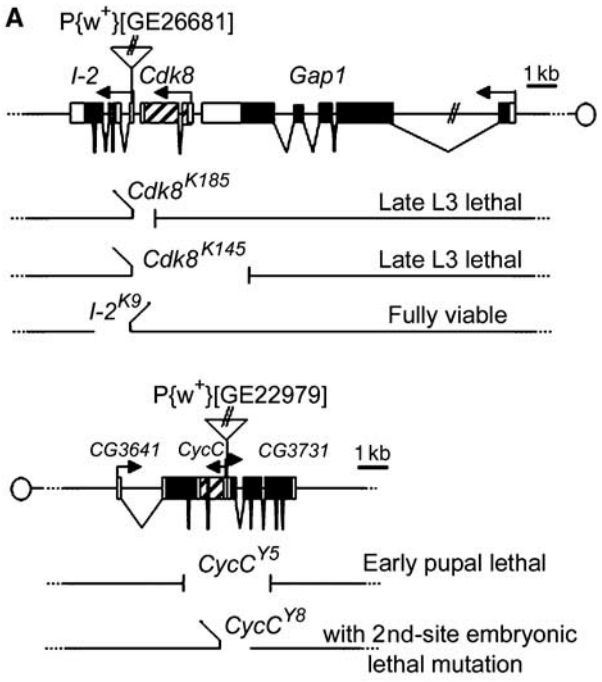
To compare functions of the four Cdk8 module components in adult development, we generated mosaic animals harboring clones of *Cdk8*$^{-}$, *CycC*$^{-}$, *Med12*$^{-}$ or *Med13*$^{-}$ cells. In light of a previously described role for *Med13* in attributing sex comb cell identity (Boube *et al*, 2000), we targeted mutant

clones to distal leg imaginal discs by expressing the Flp recombinase under the control of *Distal-less* (*Dll*) regulatory sequences (*Dll*>Flp; see Material and methods). In initial essays, we used the 'Minute' technique of growth enhancement (Morata and Ripoll, 1975) to generate large mutant clones covering the majority of the imaginal discs by the end of larval development. The resulting adult legs were severely stunted, but were less affected for $Cdk8^{-}$ and $CycC^{-}$ than for $Med12^{-}$ and $Med13^{-}$ (not shown). As the severity of the phenotypes rendered interpretation difficult, we repeated these experiments without growth enhancement. In these conditions, numerous mutant clones were systematically observed in larval leg discs, and resulted in tarsal segmentation defects in all three pairs of adult legs for each of the four subunits.

The results presented in Figure 3 summarize the effects of Cdk8 module mutants on male prothoracic (T1) distal legs. The basitarsus of a wild-type male T1 leg normally displays a sex comb composed of an aligned row of about 11 thick and darkly pigmented bristles, or teeth (Figure 3C). All four single mutants led to sex comb defects at similar frequencies (63–85%; ⩾47 legs analyzed for each genotype; Figure 3A and D–G). However, two distinct classes of defects could be discerned. For $Cdk8^{-}$ and $CycC^{-}$ clones, the mean number of sex comb teeth was slightly elevated (12 and 13, respectively; Figure 3A). However, nearly half of the mutant samples also presented at least three discontinuities in the normal tooth alignment, leading to irregularly grouped, 'fragmented' sex combs (44 and 43%, respectively; Figure 3, A and D–E). By contrast, $Med12^{-}$ and $Med13^{-}$ clones led to slightly reduced (mean = nine teeth) but mostly aligned sex combs (Figure 3, A and F–G).

Regarding leg organization, mutant clones for the four genes differentially affected overall size as well as formation of the joints separating the five tarsi along the proximo-distal axis (Figure 3B and D–G). $Med12^{-}$ and $Med13^{-}$ clones provoked strong distal leg shortening, whereas $Cdk8^{-}$ and $CycC^{-}$ showed little effect (Figure 3, compare panels F–G and E–D). The most proximal joint, separating the first and second tarsi, was affected in a majority of T1 legs for all four genotypes (53–94% defective or deleted). In the three more distal joints, however, the effects of $Med12^{-}$ and $Med13^{-}$ clones were markedly stronger (92–100%) than $Cdk8^{-}$ and $CycC^{-}$ (4–36%).

Taken together, these data lead to several conclusions. First, all four genes encoding subunits of the putative Cdk8 module are required for localized functions in normal leg development. Second, Cdk8 and CycC have indiscernible mutant phenotypes, indicating that they function as obligatory partners *in vivo* as expected for a specific Cdk–cyclin pair. Similarly, the identical mutant leg phenotypes for Med12 and Med13 reinforce the interpretation that they also function together as a pair, as previously reported for the eye and the wing (Treisman, 2001; Janody *et al*, 2003). Third, the mutant phenotypes owing to *Cdk8–CycC* differ from those of *Med12–Med13*. This result is contrary to our expectations if these



B

Genotype	% Adult viability (observed/expected)
K185/K185	0
K145/K145	0
Ub-Cdk8$^{1.14}$*/+ ; K185/K185*	104 (49/47)
I-2^{K9}*/I-2*^{K9}	98 (41/42)
Y5/Y5	0
Y5/Y8	0
arm>CycC/+ ; Y5/Y5	6 (7/121)
arm>CycC/+ ; Y5/Y8	4 (4/96)
arm>CycC/Ub-CG3731$^{F41.2}$ *; Y5/Y5*	10 (6/58)
arm>CycC/Ub-CG3731$^{F41.2}$ *; Y5/Y8*	5 (3/63)
arm>CycC/arm>CycC ; Y5/Y5	69 (56/81)

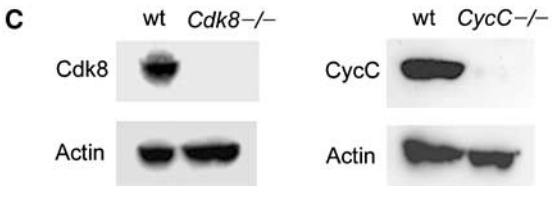


Figure 2 Isolation and characterization of null alleles of *Cdk8* and *CycC*. (**A**) Molecular organization of the *Cdk8* (upper part) and *CycC* (lower part) genomic regions and characterization of new deletion alleles. The triangles symbolize the positions of the viable *P*[*w*+] element insertions used to generate the chromosomal deficiencies. The 5′-to-3′ orientations of annotated transcription units and their exon–intron organizations are shown. Open boxes correspond to 5′- and 3′-UTRs; hatched or filled (for *Cdk8*/*CycC* or nearby genes, respectively) boxes to protein coding sequences. The broken lines below the gene structures indicate the extent of the $Cdk8^{K185}$, $Cdk8^{K145}$, $I\text{-}2^{K9}$, $CycC^{Y5}$ and $CycC^{Y8}$ deficiencies (Supplementary data), determined by DNA sequencing. The major lethality period associated with each deficiency is indicated. (**B**) Adult lethality of *Cdk8*, *I-2* or *CycC* mutant alleles and phenotypic rescue with various transgenic constructs and allele combinations (Supplementary data). For each genotype analyzed, the percentage of observed over expected adult progeny is shown, where the expected value is expressed relative to observed viable adults from balancer-marked heterozygous sibling classes. Note that $I\text{-}2^{K9}$ homozygotes are fully viable, morphologically normal adults. (**C**) Western blot analyses of Cdk8 and CycC proteins from wild-type, homozygous $Cdk8^{K185}$ or $CycC^{Y5}$ larval tissues, using polyclonal Cdk8, CycC or actin (internal loading control) antibodies. No wild-type protein was detected in mutant tissues, indicating that the maternal products have been largely exhausted 5 days after egg deposition. In the case of $Cdk8^{K185}$, the predicted truncated form in homozygous mutants was undetectable, even after long exposures (not shown).

A	*n*	Abnormal sex comb (%)	Mean tooth nb	Sex comb disruptions (%)		
				None	1–2	⩾3
Cdk8^{-}	54	63	12	37	18	44
CycC^{-}	47	75	13	32	26	43
Med13^{-}	76	85	9	34	56	11
Med12^{-}	59	83	9	36	54	10
Cdk8^{-} *Med12*$^{-}$	63	84	9	44	46	10

B	*n*	Abnormal tarsal joints (%)			
		ts1–ts2	ts2–ts3	ts3–ts4	ts4–ts5
Cdk8^{-}	55	60	11	4	36
CycC^{-}	47	53	9	11	19
Med13^{-}	72	83	90	93	92
Med12^{-}	63	94	95	100	100
Cdk8^{-} *Med12*$^{-}$	63	87	94	95	95

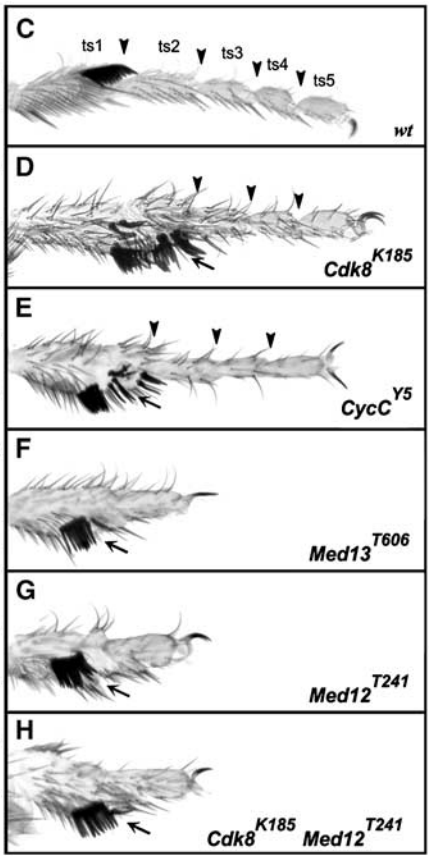


Figure 3 Comparative clonal analyses of the four Cdk8 module subunits in distal leg differentiation. (**A, B**) Sex comb abnormalities (A) and tarsal fusions (B) were quantified in the T1 legs of mosaic males harboring single mutant clones for *Cdk8*$^{-}$, *CycC*$^{-}$, *Med12*$^{-}$ or *Med13*$^{-}$, or double mutant *Cdk8*$^{-}$ *Med12*$^{-}$ clones. In all cases, mitotic clones were generated in distal precursor cells using *Dll*>Flp. For each genotype, the number of T1 legs analyzed is indicated (n). In (A), the percentage of abnormal sex combs and the mean number (nb) of teeth per leg are shown. Concerning the analysis of sex comb defects, three phenotypic classes of integrity and organization were distinguished, and are expressed as disruptions in the comb: no disruption, 'none' (e.g. panel C); one or two discontinuities, 'mild' (e.g. panels F–H); three or more discontinuities in tooth alignment, 'fragmented' (e.g. panels D and E). In (B), each of the articulated junctions separating the five tarsal segments (ts1–ts5) was counted as abnormal when the specific joint was lacking or incomplete. (**C–H**) Representative tarsi of male T1 legs from wild-type (C) or mosaic flies harboring clones of *Cdk8*$^{-}$ (D), *CycC*$^{-}$ (E), *Med13*$^{-}$ (F), *Med12*$^{-}$ (G) or *Cdk8*$^{-}$ *Med12*$^{-}$ (H) cells. Normal tarsal joints and misaligned sex-comb teeth are indicated by arrowheads and arrows, respectively.

module components serve exclusively to regulate Cdk8 kinase activity as in *S. cerevisiae*. These observations thus strongly suggested the possibility that the Cdk8–CycC and Med12–Med13 pairs have independent functions in *Drosophila*.

To ask whether the *in vivo* effects of the Cdk8–CycC pair or of its molecular partners Med12–Med13 on tarsal segmentation reflect Cdk8 module function, we tested the epistatic relationships between the two pairs. Taking advantage of the fact that *Cdk8* and *Med12* are on the same chromosome arm, we generated the double mutant and compared the distal leg phenotypes of *Cdk8*$^{-}$ *Med12*$^{-}$ clones with those due to single mutant clones (see Materials and methods). The effects provoked by *Cdk8*$^{-}$ *Med12*$^{-}$ double mutant clones were indistinguishable from *Med12*$^{-}$ alone but distinct from their *Cdk8*$^{-}$ counterparts, both for sex comb differentiation and for overall leg structure (Figure 3A–B, compare panels 3D and G with H). This indicates that *Med12* is epistatic to *Cdk8*. Clones of *Cdk8*$^{-}$ cells lead to a characteristic fragmented sex comb only in the presence of normal *Med12* function. This observation supports the interpretation that the Cdk8–CycC pair functions together with the Med12–Med13 pair within the Cdk8 module in this developmental program. Conversely, the distal tarsal defects specific to *Med12*$^{-}$ clones are not modified in *Cdk8*$^{-}$ *Med12*$^{-}$ double mutant clones. This shows that *Med12* does not require *Cdk8* to exert its effects there. These data thus support the existence of both linked and independent functions for the Cdk8 module components.

Med12 and Med13 differentially regulate gene expression in tarsal development compared with Cdk8 and CycC

To identify molecular targets of the Cdk8 module subunits, we sought leg patterning genes whose expression in L3 imaginal discs is altered in mutant clones. Tarsal segmentation is specified through a relatively well-described genetic cascade (Kojima, 2004). Among a number of candidate genes examined, the expression patterns of most regional markers were unaffected in clones of mutant cells. Such markers included *decapentaplegic* (*dpp*), *wingless*, *Dll* and *dachshund* (*dac*). By contrast, regional expression of the *bric-à-brac-2* (*bab2*) gene was markedly altered. The *bab2* gene encodes a BTB-class transcription factor that is required for elaboration of the proximo-distal leg axis and is expressed in concentric rings of cells prefiguring each tarsal segment (Couderc *et al*,

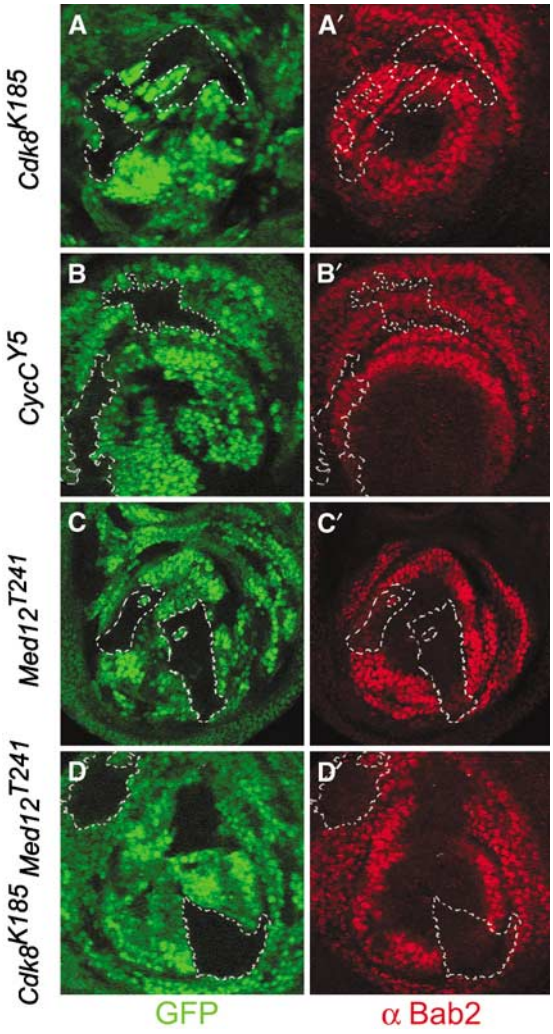


Figure 4 *Med12*, but not *Cdk8* or *CycC*, activity is required for *bab2* expression in leg discs. (**A–D**) Leg discs from L3 harboring clones of *Cdk8*$^{-}$ (A), *CycC*$^{-}$ (B), *Med12*$^{-}$ (C) or *Cdk8*$^{-}$ *Med12*$^{-}$ (D) cells. Mitotic clones were generated using *Dll*>Flp. The left panels show clones of mutant cells (black, GFP^{-}; representative examples are circled) in a background of wild-type (GFP+, green) cells. The right panels show *bab2*-expressing cells (red), as revealed by anti-Bab2 antibody staining. Nuclear Bab2 is autonomously reduced in *Med12*$^{-}$ single or *Cdk8*$^{-}$ *Med12*$^{-}$ double mutant cells, but is not modified in *Cdk8*$^{-}$ or *CycC*$^{-}$ cells.

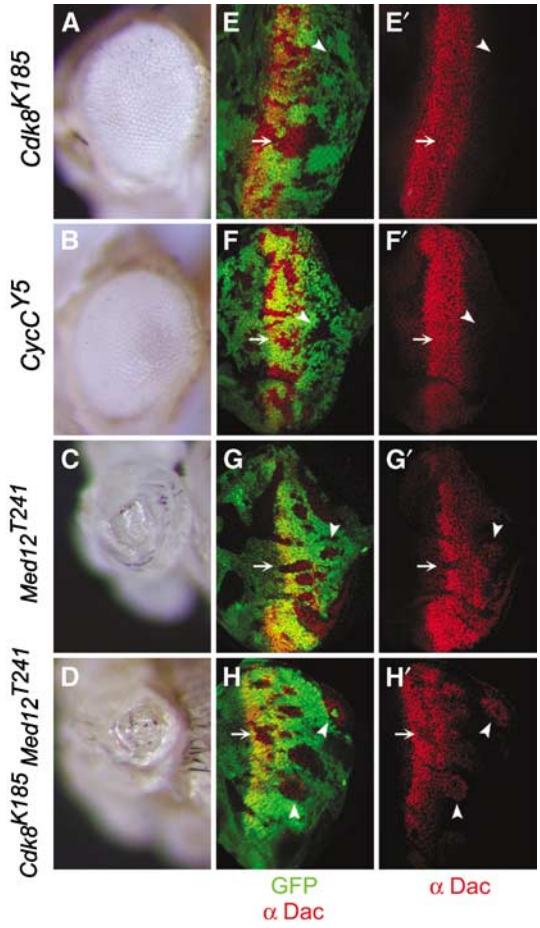


Figure 5 *Cdk8* and *CycC* activities are dispensable for early eye differentiation, as opposed to *Med12* and *Med13*. (**A–D**) Adult eyes almost entirely composed of *Cdk8*$^{-}$ (A), *CycC*$^{-}$ (B), *Med12*$^{-}$ (C) or *Cdk8*$^{-}$ *Med12*$^{-}$ (D) cells. Mitotic clones were induced in the developing eye discs, using an *ey*-Flp construct and in a *Minute* background to confer a growth advantage to mutant cells. (**E–H**) Eye discs of L3 harboring clones of *Cdk8*$^{-}$ (E), *CycC*$^{-}$ (F), *Med12*$^{-}$ (G) or *Cdk8*$^{-}$ *Med12*$^{-}$ (H) cells. Clones were generated without *Minute* background. The middle panels show clones of mutant cells (GFP^{-}, black). The middle (E–H) and right (E′–H′) panels show *dac*-expressing cells (in red), as revealed by anti-Dac antibody staining. Nuclear Dac protein is normally limited to a broad band of cells corresponding to the MF. *Cdk8*$^{-}$ or *CycC*$^{-}$ cells express Dac normally. In contrast, *dac* is downregulated in *Med12*$^{-}$ or *Cdk8*$^{-}$ *Med12*$^{-}$ cells within the MF (arrows) and upregulated more posteriorly (arrowheads).

2002). Mutant clones for *Med12* or *Med13* in this region cell-autonomously downregulated *bab2* expression (Figure 4C, and not shown). In contrast, *bab2* expression was not affected either for small clones of *Cdk8*$^{-}$ or *CycC*$^{-}$ cells (Figure 4A–B) or for large, Minute-enhanced clones that occupied nearly the entire leg disc (not shown). These data establish that both Med12 and Med13, but not Cdk8 or CycC, are required to activate and/or maintain *bab2* expression. Accordingly, *bab2* is downregulated in *Cdk8*$^{-}$ *Med12*$^{-}$ double mutant cells as for *Med12*$^{-}$ alone (Figure 4, compare D and C).

Med12–Med13 act independently of Cdk8–CycC during early eye differentiation

To examine Cdk8 module functions in another adult tissue, we generated clones of *Cdk8*$^{-}$, *CycC*$^{-}$, *Med12*$^{-}$, *Med13*$^{-}$ or *Cdk8*$^{-}$ *Med12*$^{-}$ cells in the developing eye. As previously reported (Treisman, 2001), large *Med12*$^{-}$ or *Med13*$^{-}$ clones failed to differentiate ommatidia (Figure 5C, and not shown). Further, *Med12*$^{-}$ or *Med13*$^{-}$ clones cell-autonomously misexpressed two early-acting eye patterning genes, *dac* and *dpp*, failing to activate these targets within the morphogenetic furrow (MF) or to repress them more posteriorly (Figure 5G–G′) (Treisman, 2001). By contrast, adults harboring large *Cdk8*$^{-}$ or *CycC*$^{-}$ clones developed full-sized eyes with mostly normal differentiated ommatidia (Figure 5A–B). Consistent with this, clones of *Cdk8*$^{-}$ or *CycC*$^{-}$ cells showed no effect on *dac* expression anywhere in the eye disc (Figure 5E–F). As for the legs (see above), the effects of *Cdk8*$^{-}$ *Med12*$^{-}$ double mutant clones were indiscernible from *Med12*$^{-}$ alone (Figure 5C–D). Misexpression of *dac* was still observed for *Cdk8*$^{-}$ *Med12*$^{-}$ clones both within and posterior

to the MF (Figure 5H–H′). We conclude that Med12–Med13 act independently of Cdk8–CycC in early eye differentiation.

Shared functions of Med12–Med13 and Cdk8–CycC in external sensory organ development

Although the results described above indicate that Med12–Med13 and Cdk8–CycC pairs can act independently in developing legs and eyes, no evidence conclusively supported shared functions. Having observed that partial rescue of *CycC*$^{-}$ mutants by transgenic constructs resulted in adults with reduced numbers of external sensory organs including macrochaetes and microchaetes (above), we examined bristle specification/differentiation for the adult notum. In preliminary experiments where mutant clones were generated in L3 larvae by heat-pulse-induced Flp recombinase, clones of *Med12*$^{-}$, *Med13*$^{-}$, *Cdk8*$^{-}$ or *CycC*$^{-}$ cells were all associated with localized loss of bristles (not shown). Macrochaete specification occurs in late L3 (Lai and Orgogozo, 2004), at a time when normal Cdk8 and CycC proteins are no longer detected in mutants (see above). We therefore analyzed in greater details the effects of representatives of each Cdk8 module pair, *Med12* and *Cdk8*, on macrochaete development. Minute-enhanced clones of *Med12*$^{-}$ or *Cdk8*$^{-}$ cells were induced in the dorsal compartment of the wing discs, coupling the *ap*-Gal4 driver (Calleja *et al*, 1996) with an *UAS*-Flp element. Whereas the notum normally show a stereotyped pattern of bristles, animals harboring large clones of *Med12*$^{-}$ or *Cdk8*$^{-}$ cells displayed extensive loss of macrochaetes (Figure 6, compare panels A–C). Taken together, these data support an action of Med12–Med13 in external sensory organ specification/differentiation that is shared with Cdk8–CycC.

To identify molecular targets coregulated by the four Cdk8 module subunits, we next examined candidate genes known to be required for peripheral nervous system (PNS) development (Lai and Orgogozo, 2004). The PNS organs are generated by asymmetric divisions from an initially specified sensory organ precursor (SOP). A specific marker for SOPs and their descendant cells is the expression of the *senseless* (*sens*) gene product, a zinc-finger transcription factor that is specifically expressed in SOPs and required for their normal development (Nolo *et al*, 2000). Sens protein expression was mostly absent from mosaic L3 wing discs in clones of *Med12*$^{-}$ or *Cdk8*$^{-}$ cells (Figure 6E–F). Taken together, our results indicate a shared role of Med12–Med13 and Cdk8–CycC pairs in normal regulation of *sens* during SOP specification/differentiation.

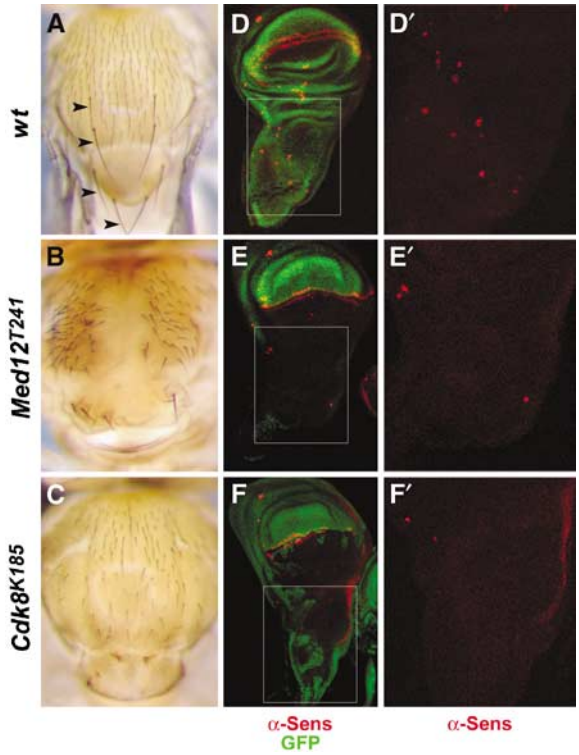


Figure 6 Med12 and Cdk8 are both required for macrochaete specification/differentiation, including for normal *sens* expression in wing discs. (**A–C**) Representative nota from wild-type (A) or mosaic adults harboring large clones of *Med12*$^{-}$ (B) or *Cdk8*$^{-}$ (C) cells. Mitotic clones were generated in dorsal wing disc cells (see below), using *ap*-Gal4 together with a *UAS*-Flp construct and in a *Minute* background to confer a growth advantage to mutant cells. In the wild type, each hemi-notum normally displays a stereotyped pattern of large bristles, or macrochaetes (some are indicated by arrowheads). Note that apart from the loss of macrochaetes, large clones for *Med12* can be accompanied by a thoracic cleft never seen for *Cdk8*. (**D–F**) Projections of confocal sections of entire L3 wing discs from wild-type (D–D′) or mosaic discs harboring large clones of *Med12*$^{-}$ (E–E′) or *Cdk8*$^{-}$ (F–F′) cells. Mutant cells are GFP^{-} (black). SOPs are revealed by anti-Sens antibody staining (in red). Boxes in (D–F) indicate the notum region, enlarged in the right panels (D′–F′). The ~20 SOPs expressing *sens* in the thoracic region of late L3 wild-type discs (Nolo *et al*, 2000) contrast with the few remaining cells accumulating nuclear Sens protein in large *Med12*$^{-}$ or *Cdk8*$^{-}$ clones.

Discussion

In this study, we have investigated the functions of the four Cdk8 module subunits in the metazoan model *Drosophila*, comparing *in vivo* defects induced by null mutants of *Cdk8*, *CycC*, *Med12* and *Med13*. Each gene is essential for the development of the organism but not for cell viability. Our observation that fly Cdk8 and CycC proteins interact with Med12 and Med13 *in vitro* and/or in yeast cells reinforces the notion of a conserved Cdk8 module deduced from whole-genome analyses (Boube *et al*, 2002). Our genetic data provide evidence that the four proteins composing the fly Cdk8 module can act together *in vivo*, as seen for the regulation of *sens* in sensory organ development. However, their divergent effects in regulating the target genes *bab2*, *dac* and *dpp* during tarsal and eye differentiation lead us to infer a functional diversification of Cdk8–CycC and Med12–Med13 into two distinct pairs possessing diverging functions within the confines of a shared MED module. Interestingly, whereas Med12 and Med13 are specifically required for a temporally restricted regulation of *dac* and *dpp* in eye disc cells, the same patterning genes are unaffected in leg disc mutant cells. This indicates that Med12–Med13 activity depends both on the target genes and the developmental context.

The prevailing view of Cdk8 module action, based on the functional comparison of its four components in the budding yeast, holds that this MED module serves exclusively to ensure regulated kinase activity (Myer and Young, 1998). Interestingly, recent work has raised the possibility of a Cdk8-independent Med13 activity situated downstream of the *S. cerevisiae* Ras/PKA signaling pathway (Chang *et al*, 2004). Our comparative analysis has revealed that *Drosophila* Med12–Med13 can act independently of Cdk8–CycC. One

possible explanation is that another Cdk and/or cyclin may partially replace these proteins within the fly 'Cdk8' module. However, whereas purified mammalian MED complexes contain the Cdk8-related subunit Cdk11 (Conaway *et al*, 2005) and human Cdk3 can interact with CycC in cultured cells (Ren and Rollins, 2004), neither Cdk11 nor Cdk3 has a counterpart in the *D. melanogaster* genome. Furthermore, the equivalent mutant phenotypes of *Cdk8* and *CycC* in our *in vivo* analysis support the idea that Cdk8 or CycC do not associate with another cyclin or Cdk. We conclude that *Drosophila* Med12–Med13 likely function independently of a Cdk kinase activity *in vivo*.

The preceding considerations lead us to deduce that Med12 and Med13 are able to associate with the core MED independently of Cdk8 or CycC. Conversely, it has been proposed that *S. cerevisiae* Med12 and Med13 are required for the stable association of the Cdk8–CycC pair to core MED (Myer and Young, 1998). Accordingly, MED from human cells depleted for Med12 also exhibits diminished Cdk8 (Kim *et al*, 2006). In the present work, both our observed binding of fly Med12 and Med13 to Cdk8 and CycC *in vitro* and the genetic dependency of *Cdk8* on *Med12 in vivo* reinforce this model. Importantly, although our data are consistent with a role of Med12–Med13 proteins in associating the Cdk8–CycC pair to core MED, they above all highlight that Med12–Med13 also act independently of Cdk8 or CycC.

The biochemical nature of this Cdk-independent activity of Med12–Med13 remains to be deciphered. It seems likely that the large Med12–Med13 pair (~500 kDa in metazoans) directly contributes to the extensive structural rearrangements occurring within MED on binding to specific activators (Taatjes and Tjian, 2004). Consistent with this idea, mammalian Med12 physically interacts with diverse transcriptional regulators (Zhou *et al*, 2002; Gwack *et al*, 2003; Kim *et al*, 2006; Zhou *et al*, 2006). Such Cdk-independent regulatory activity of Med12 and Med13 may directly impact the interaction of core MED with PolII. We speculate that kinase-independent Med12–Med13 activities may have contributed extensively during evolution to regulate and diversify cell differentiation processes.

Materials and methods

Recombinant protein expression and GST pull-down assay

Each pGEX plasmid (Leclerc *et al*, 1996) was transformed into *Escherichia coli* BL21 (Novagen). GST alone and GST fusion proteins were purified as recommended by GE Healthcare. The GST and GST fusion proteins bound to glutathione–Sepharose (GS) beads were equilibrated in 10 mM Tris (pH 8), 100 mM NaCl, 10 mM KCl, 1 mM EDTA, 100 μg/ml bovine serum albumin (BSA), 1% Nonidet P-40 (NP-40) and 1 mM dithiothreitol (DTT). In some experiments, slightly less stringent conditions (i.e. 0.5% NP-40) were used. ^{35}S-labeled proteins were produced by coupled *in vitro* transcription/translation (Promega), incubated with GST, GST–Cdk8 or GST–CycC beads (with ~5 μg GS-bound proteins per assay) for 1 h at 4°C and washed five times in 20 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA, 1% NP-40 and 1 mM DTT. Bound proteins were eluted with an excess of reduced glutathione and radiolabeled polypeptides analyzed by SDS–PAGE followed by fluorography using Amplify (GE Healthcare).

Yeast two-hybrid assay

The entire open reading frames of *Cdk8*, *CycC*, *Med12* and *Med13* (Leopold and O'Farrell, 1991; Leclerc *et al*, 1996; Treisman, 2001) were cloned into pAS2 and pACT2 vectors (details available upon request). Two-hybrid assays were performed in Y190 (*MAT***a**, *gal4*, *gal80*, *his3*, *trp1-901*, *ade2-101*, *ura3-52*, *leu2-3*, *112 URA3*::*GAL1*::*lacZ*, *LYS2*::*GAL4(UAS)*::*HIS3*, *cyh*^{R}). The interaction of each pair of *Drosophila* Cdk8 module subunits was tested by cotransformation of the pAS2-Med plasmids encoding the G_{BD} fusions with the pACT2-Med plasmids encoding the G_{AD} fusions. Transformants were selected on SC-Leu-Trp plates. β-Galactosidase activity was revealed by an X-Gal overlay assay as previously described (Werner *et al*, 1993).

Western blotting

For each genotype, total protein extracts from ten L3 ventral nerve cords plus associated imaginal discs boiled for 5 min in 2 × Laemmli sample buffer were subjected to Western blot analyses (10 or 12% SDS–polyacrylamide gels). Apparent masses were determined by comparison with prestained SDS–PAGE molecular weight standards (NEB Biolabs). Rabbit polyclonal antibodies directed against human Cdk8 (1/200) or *Drosophila* CycC (1/100) (Leclerc *et al*, 1996) were kindly provided by P Leopold. The mouse monoclonal antibody directed against actin (Mab1501 from Chemicon International Inc.) was kindly provided by B Raynaud-Messina (1/10 000).

Isolation of Cdk8 and CycC mutants

P[w^{+}] inserts near *Cdk8* and *Cyc*C (termed GE26681 and GE22979, respectively) were acquired from GenExel Inc. Mutant chromosomes were generated by mobilizing each *P* element with the Δ2–3 *P* transposase source (FlyBase) and white-eyed flies selected. Recessive lethal chromosomes were subjected to complementation tests with large deficiencies removing the region of *Cdk8* [*Df(3L)AC1)*] or *CycC* [*Df(3R)Exel6172*] (see FlyBase). Five selected mutant chromosomes, *Cdk8*^{K185}, *Cdk8*^{K145}, *I-2*^{K9}, *CycC*^{Y5} and *CycC*^{Y8}, were characterized molecularly by PCR and DNA sequencing.

Transgenic constructs and phenotypic rescue experiments

p*UAS*-CycC, p*Ub*-Cdk8, p*Ub*-CG3731 and p*Ub*-CG3641 were generated from full-length cDNA inserts, kindly provided by P Leopold (Leopold and O'Farrell, 1991; Leclerc *et al*, 1996) or obtained from the Berkeley Drosophila Genome Project (RH09020 and RE07395, for *CG3731* and *CG3641*, respectively), and used for generating transgenic lines (details available upon request). *Cdk8*^{K185} and *Cdk8*^{R145} homozygotes were rescued by a single copy of *Ub*-Cdk8 (insertion 1.14, chromosome 2). For *CycC*^{Y5}, an *arm*-Gal4 insert (line 11; FlyBase) recombined with our *UAS*-CycC insertion wdE onto chromosome 2 (*arm* > *CycC*) was used as a source of wild-type CycC.

Genetic mosaics

Mitotic clones were generated using the Flp-FRT system (Xu and Rubin, 1993). *Cdk8*^{K185} and *CycC*^{Y5} were recombined onto chromosomes carrying FRT80B or FRT82B, respectively. Flp recombinase was expressed in the developing eye disc using an eyeless (*ey*)-Flp construct on the X-chromosome (Treisman, 2001); in the distal ventral appendages using the *Dll*^{EM212}-Gal4 insert (Gorfinkiel *et al*, 1997) recombined with a *UAS*-Flp construct on chromosome 2 (Bloomington *Drosophila* Stock Center (BDSC)); or in the dorsal wing disc compartment using the *ap*-Gal4 driver (Calleja *et al*, 1996) in the presence of the same *UAS*-Flp insert. Clones were visualized using the *Ub*-GFP, FRT80B or FRT82B, *Ub*-GFP chromosome (BDSC). To generate large clones in the adult eye, leg or notum by the 'Minute' technique (Morata and Ripoll, 1975), *M(3)RpS17*, *Ub*-GFP, FRT80B or FRT82B, *Ub*-GFP, *M(3)RpS3* recombinant chromosomes were used.

Histology, whole-mount immunostaining of imaginal discs and adult cuticle analysis

Histology and antibody staining were performed as described (Boube *et al*, 2000), using mouse anti-Dac (1/100; from Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Wg (1/100; from DSHB), mouse anti-Dll (1/500; from I Duncan), guinea-pig anti-Sens (1/1000; from H Bellen) or rat anti-Bab2 (1/5000; from J-L Couderc). Adult legs were mounted in Hoyer's medium and examined with a light microscope.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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